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Cometabolic Degradation of Trichloroethene by *Rhodococcus* sp. Strain L4 Immobilized on Plant Materials Rich in Essential Oils▽†

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The cometabolic degradation of trichloroethene (TCE) by *Rhodococcus* sp. L4 was limited by the loss of enzyme activity during TCE transformation. This problem was overcome by repeated addition of inducing substrates, such as cumene, limonene, or cumin aldehyde, to the cells. Alternatively, *Rhodococcus* sp. L4 was immobilized on plant materials which contain those inducers in their essential oils. Cumin seeds were the most suitable immobilizing material, and the immobilized cells tolerated up to 68 μM TCE and degraded TCE continuously. The activity of immobilized cells, which had been inactivated partially during TCE degradation, could be reactivated by incubation in mineral salts medium without TCE. These findings demonstrate that immobilization of *Rhodococcus* sp. L4 on plant materials rich in essential oils is a promising method for efficient cometabolic degradation of TCE.

Various bacteria have been reported to degrade trichloroethene (TCE) aerobically via cometabolic degradation with broad-substrate-specificity enzymes (2). However, TCE cometabolic degradation is considered an unsustainable process due to cytotoxicity, inhibition, or inactivation of TCE-degrading enzymes. These phenomena have been observed in studies using both whole cells and purified enzymes, including soluble methane monooxygenases from *Methylosinus trichosporium* OB3b (9) and *Nitrosomonas europaea* (13), toluene 2-monooxygenase from *Burkholderia cepacia* G4 (19, 27), toluene dioxygenase (TDO) from *Pseudomonas putida* F1 (15, 18), and butane-oxidizing bacteria, i.e., *Pseudomonas butanovora*, *Mycobacterium vaccae*, and *Nocardioides* sp. CF8 (11). Nevertheless, the addition of an inducer or growth substrate can maintain TCE cometabolic degradation. For example, the TCE-degrading activity of *P. putida* F1 toluene dioxygenase was restored after adding benzene, cumene, or toluene to displace TCE and its reactive intermediates from the enzyme active site (18). Arp et al. (2) suggested that the rate of enzyme maintenance and recovery depended on the extent of inactivation and the balance of TCE and inducer/growth substrate concentrations.

Plant essential oils and their components, such as citral, limonene, cumene, and cumin aldehyde, have been found to induce TCE degradation in *Rhodococcus* sp. L4 (24). However, the removal of TCE by this bacterium was effective only for a short period. The impacts of TCE on *Rhodococcus* spp. and their enzymes have not been studied in detail, even though many bacteria of this genus exhibited high TCE-degrading activities (i.e., *Rhodococcus erythropolis* JE 77, *R. erythropolis* BD2, *Rhodococcus* sp. Sm-1, and *Rhodococcus* sp. Wrink) (5, 6, 7, 16). This study therefore investigated the changes in TCE-degrading activity of *Rhodococcus* sp. L4 cells and TDO during exposure to TCE. Two enzyme maintenance approaches were evaluated, namely, repeated addition of essential oil components to the system and immobilization of the bacterial cells on plant material rich in essential oils. Immobilized microorganisms are generally capable of degrading pollutants at a higher initial concentration and for a longer period than those of free cells (21, 23), possibly because the microbial cells are protected from environmental stress and toxic compounds (3). In this study, the plant materials were used to provide a solid surface for bacterial attachment and a continuous source of essential oils for inducing TCE-degrading enzymes. Our results show that the repeated addition of limonene, cumene, or cumin aldehyde enhances TCE degradation and that bacteria immobilized on cumin seeds are able to maintain their TCE-degrading activity.

MATERIALS AND METHODS

Chemicals, materials, and bacterial culture. TCE (99.5%), cumene (99.0%), *R*-(+)-limonene (96.0%), and cumin aldehyde (4-isopropyl benzaldehyde) (90.0%) were obtained from Fluka, Switzerland. All other chemicals used in this study were of analytical grade and were of the highest purity available. Plant materials, including cumin seeds, lemongrass leaves, orange peels, and loofah (*Luffa cylindrica*) sponges, were purchased in one batch from a local market in Bangkok, Thailand. Granular activated carbon was purchased from Merck. *Rhodococcus* sp. L4 is deposited at the Microbiological Resources Center, Thailand Institute of Scientific and Technological Research, under accession number TISTR 1542. The bacteria were maintained by being cultured on mineral salts (MS) agar incubated in a glass box equilibrated with toluene vapor at room temperature. The MS medium consisted of 10 mM K_2HPO_4, 3 mM $NaH_2PO_4 \cdot 7H_2O$, 10 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $Ca(NO_3)_2$, 0.01 mM $Fe(NO_3)_3 \cdot 7H_2O$, and the trace elements described by Focht (8).

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Effects of TCE and essential oil components on activity of *Rhodococcus* sp. L4 cells. Preparations of cumene-, limonene-, cumin aldehyde-, and cumin oil-induced cell inocula and resting-cell assays of TCE degradation were carried out as previously described (24). Generally, the experiments contained resting-cell suspensions and TCE in a 22-ml-headspace vial sealed with a Teflon-faced silicone septum and an aluminum crimp cap. Oxygen was provided by leaving a 90% air headspace in the vials prior to sealing. The amount of oxygen in air at the end of the study, as detected by gas chromatography-thermal conductivity detector (GC-TCD), was approximately 14 to 29%, which implies that sufficient oxygen was available during TCE biodegradation. The reactions were stopped by adding 1 drop of 10 M H_2SO_4. The control set consisted of heat-killed cells containing TCE, which represented abiotic loss during the assay.

The TCE concentration varied from 2 to 68 μM. The aqueous concentrations of TCE in liquid-gas systems at 30°C were calculated with a dimensionless Henry's constant of 0.49 (10). The total TCE mass that remained in the system was calculated from the amounts of TCE in both the aqueous and gaseous phases. All experiments were performed in triplicate. To enhance the TCE-degrading ability of *Rhodococcus* sp. L4, stock solutions of the essential oil components prepared in *N*,*N*-dimethylformamide were added to the cells during TCE degradation assays to give a final nominal concentration of 80 μM cumin aldehyde or 200 μM limonene or cumene, as described by Suttinun et al. (24). The amounts of remaining TCE and essential oil components were measured at different time intervals.

Effects of TCE on partially purified TDO. The enzyme involved in initial TCE degradation by toluene/cumene-induced *Rhodococcus* sp. L4 was preliminarily characterized as TDO (E. Luepromchai, unpublished data), the enzyme was partially purified, and its activity in the presence of various TCE concentrations (20, 40, 60, and 80 μM) was tested. To obtain larger amounts of cells for enzyme extraction, *Rhodococcus* sp. L4 was cultured by transferring a 60-ml late-log-phase inoculum to a 2-liter Duran bottle containing 540 ml of MS medium and 3 mmol $liter^{-1}$gas-phase cumene and incubating it for 60 h at 180 rpm at room temperature. The cumene vapor was supplied by adding 600 μl cumene to a sterilized Eppendorf tube that hung at the top of the culture bottle. Crude protein was extracted from the cell pellets by using BugBuster Master Mix reagent (Novagen) according to the manufacturer's instructions, by resuspending 1.5 g of cell paste in 5 ml of reagent. The cell-free crude extract was filtered through a 0.2-μm membrane and stored at −20°C until needed. The partial purification of crude protein extract (0.95 mg ml^{-1}) was carried out by ammonium sulfate precipitation according to the method of Lange and Wackett (15). The protein pellet was resuspended in 100 mM Tris-HCl buffer (pH 7.5) containing 10% ethanol and 10% glycerol (TEG buffer), supplemented with 1 mM dithiothreitol (DTT), at a ratio of 1 g pellet per 2 ml buffer. The highest TDO activity was obtained with the fraction that precipitated 0 to 60% ammonium sulfate.

The effect of TCE on TDO activity was measured in a 3-ml reaction mixture which contained partially purified protein, all ingredients for TDO assay stated below, and 0 to 80 μM TCE. Recovery of TDO activity was studied by placing a sample containing 80 μM TCE in a 14,000-molecular-weight-cutoff dialysis bag and submerging it in 100 ml cold TEG buffer to remove the nonreacted TCE and degradation intermediates. The amount of TCE outside the dialysis bag was analyzed to ensure that equilibrium was reached. After 3 h of incubation, the buffer was replaced and the incubation was continued for another 3 h. The solution inside the dialysis bag was collected and assayed for the recovery of TDO activity. Sample measurements were made in duplicate.

TDO activity was assayed by a method modified from the work of Jenkins and Dalton (14), based on the measurement of a yellow dye (indoxyl) by spectrophotometry (A_{400}) (Specord 40 Plus WinASPECT software; Analytik Jena AG). Indoxyl is formed via oxidation of indole to indigo by TDO. The reaction was performed in a quartz cuvette containing 3 ml of 100 mM Tris-Cl buffer, 0.1 mM $FeSO_4$, 2 mM NADH, 0.46 mg protein, and 0.2 mM indole. The sample was mixed thoroughly before the absorbance at 400 nm was measured against a blank containing all ingredients except indole. Protein was quantified by the Bradford assay (Bio-Rad) according to the manufacturer's instructions.

Immobilization of bacteria on plant materials. Three plant materials rich in essential oils, namely, cumin seeds, orange peels, and lemongrass leaves, were screened as immobilizing supports for *Rhodococcus* sp. L4. Dried plant materials were ground and sieved to a 1- to 2-mm particle size. The materials were sterilized by being autoclaved three times consecutively. Cell suspensions of toluene-induced *Rhodococcus* sp. L4 were prepared as described previously (24). The immobilization was carried out by an attachment technique in which 1 g of sterilized plant material was added to flasks containing 50-ml cell suspensions in MS medium (optical density at 600 nm [OD_{600}] = 1.0) and incubated at 130 rpm at room temperature for 4 days. The immobilized cultures were washed with 10 ml MS medium, filtered through sterilized filter paper (Whatman no. 41) for removal of unattached cells, and air dried in a sterile hood. Loofah sponge and activated carbon were used as control materials without essential oils. Another set of controls with uninoculated material and killed immobilized cells (with 2 to 3 drops of 10 M H_2SO_4) was used to determine the abiotic loss of TCE, i.e., the amount of TCE adsorbed on bacterial cells and on the materials. After initial testing, cumin seeds were selected as the immobilizing support for further experiments because they showed the highest capability for TCE degradation and stability in repeated applications. The remaining amounts (wt/wt) of the major oil components terpinene, pinene, cymene, cumin aldehyde, and cumene in cumin seeds after the immobilization process were 16%, 9%, 15%, 6%, and 0.2%, respectively.

To monitor bacterial attachment, the immobilized samples were examined by a scanning electron microscope (JEOL model JSM-5410LV). The amount of exopolysaccharides (EPS) which ensured a strong attachment of bacteria to materials during immobilization (21) was determined by an Alcian Blue adsorption method modified from the work of Vandevivere and Kirchman (26). The bacteria were incubated with cumin seeds of various sizes (500 μm to 2 mm) for different times to determine the optimum incubation time for stable cell attachment. The assay for production of EPS was performed every 24 h by transferring 100 μl of 1% Alcian Blue solution in 3% acetic acid into flasks containing 10 ml MS medium and 1 g immobilized cells. The samples were shaken at 80 rpm for 15 min. After removal of immobilized cells, the absorbance of the supernatant was measured at 606 nm. The decline in the absorbance relative to that of the control, i.e., cumin seeds without cells plus dye solution, was a measure of the amount of exopolysaccharides produced.

The number of attached bacteria was analyzed after the bacteria were extracted from cumin seeds by a procedure modified from the work of Pattanasupong et al. (22). The immobilized matrices were rehydrated in MS medium for 3 min, sonicated (Clifton ultrasonic bath) for 2 min, and shaken vigorously on a vortex mixer for another 2 min. This process was repeated twice. The cell suspension was used to determine the number of bacteria by the plate count technique, and cell dry weight was calculated from the mass difference between a preweighed dry crucible with 10 ml of MS medium added and that with 10 ml of bacterial culture added after both sets were incubated overnight at 103 to 105°C (24). Samples were measured in duplicate.

TCE degradation by immobilized cells. The assay mixtures for the determination of TCE degradation by immobilized cells consisted of 0.02 g of immobilized materials in 22-ml-headspace vials containing 160 nmol TCE (14 μM initial aqueous concentration) in 2 ml MS medium. To observe the effect of TCE concentration, the TCE concentration in another experiment was varied from 20 to 800 nmol TCE (2 to 68 μM initial aqueous concentration). Semicontinuous TCE degradation was performed by repeatedly adding 160 nmol TCE to the vials on days 4, 5, and 6. The aqueous concentrations of TCE prior to and after each TCE addition were <50 μM, which did not cause bacterial inhibition. The control sets consisted of uninoculated samples and samples with killed immobilized cells to determine TCE loss by abiotic processes. To reactivate the bacteria after TCE degradation, the immobilized cells were washed with MS medium before being placed in a 250-ml Erlenmeyer flask containing 50 ml MS medium per gram of material. The flasks were incubated at room temperature with shaking at 130 rpm for 12 to 24 h before they were reused to determine TCE biodegradation. All of the experiments were performed in triplicate.

Analysis of TCE and essential oil components. The amounts of TCE and essential oil components were analyzed by headspace gas chromatography (Perkin-Elmer TurboMatrix automated headspace sampler with a Clarus 500 GC) with a flame ionization detector (headspace GC-FID) and an HP-5 (5% phenyl methyl siloxane) fused-silica capillary column (30 m × 0.32-mm internal diameter; thickness, 0.25 μm). To achieve equilibration between the gas and liquid phases, the sample vials were heated to 93°C for 30 min before GC analysis. GC conditions were those described by Suttinun et al. (24). The retention time of TCE was 1.78 min, while cumene, limonene, and cumin aldehyde showed retention times of 4.17, 5.69, and 8.90 min, respectively. External standard quantitative calibrations were used for the calculation of TCE and essential oil component concentrations.

RESULTS

Effects of TCE on *Rhodococcus* sp. L4 and its TDO activity. TCE-degrading efficiency of cumene-induced cells increased (1 to 120 nmol TCE) with TCE concentrations of 2 to 50 μM, beyond which the degrading ability decreased moderately

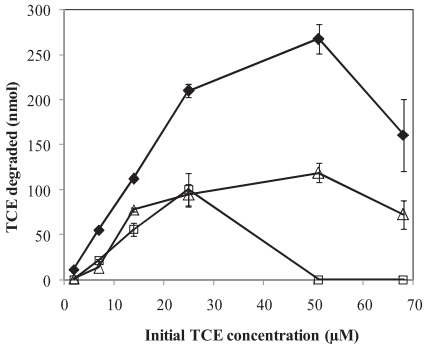

FIG. 1. Effect of TCE concentration on *Rhodococcus* sp. L4 cells after induction by cumene (△) or cumin essential oil (□) or on cumin seed-immobilized cells (◆). The initial TCE concentration was varied from 2 μM to 68 μM. TCE-degrading activities of suspended and immobilized cells were analyzed after 8 h and 5 days of incubation. The mass of suspended cells was 0.8 mg cell dry weight (OD_{600} = 1), while the immobilized cells contained 0.55 mg cell dry weight on 0.02 g cumin seeds.

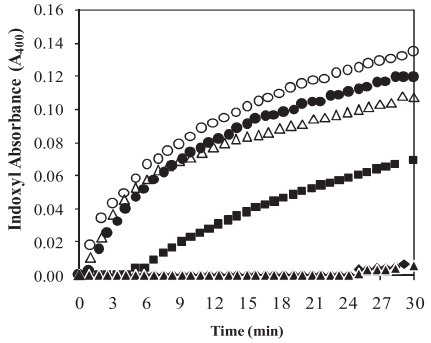

FIG. 2. Effect of TCE concentration on TDO activity of cumene-induced *Rhodococcus* sp. L4. TDO activities in samples without TCE (○) or with 20 μM (●), 40 μM (■), 60 μM (◆), or 80 μM (▲) TCE are presented. TDO activity in the sample after removal of TCE by dialysis is also shown (△).

(Fig. 1). Cumin oil-induced cells exhibited similar behavior, but they were able to degrade TCE at lower concentrations (<50 μM). The amount of TCE degraded by the suspended cells was 0.1 to 120 nmol (Fig. 1). These results indicated that *Rhodococcus* sp. L4 was inhibited by high concentrations of TCE and that the extent of inhibition depended on the type of inducer. TDO activities in all TCE-containing samples were lower than that in the sample without TCE (Fig. 2). In the absence of TCE, TDO showed a maximum specific activity for indole oxidation (0.057 A_{400} unit min^{-1} mg $protein^{-1}$), whereas the activity decreased in the presence of TCE, to 0.049 to 0.000 A_{400} unit min^{-1} mg $protein^{-1}$ in the first 9 min, which corresponds to 15 to 100% activity loss. However, TDO activity increased significantly after the removal of TCE by dialysis (Fig. 2). The recovered TDO had a specific activity of 0.052 A_{400} unit min^{-1} mg $protein^{-1}$, which was slightly lower than that of the sample without TCE and corresponded to 90% recovery.

Repeated addition of essential oil components to enhance TCE degradation by *Rhodococcus* sp. L4. When the essential oil component was added only at the beginning, all induced cells were able to degrade TCE after a 1-h lag period (Fig. 3a, b, and c). Meanwhile, the added oil component was degraded readily. TCE-degrading activities of these bacteria were comparable to those without oil addition in the work of Suttinun et al. (24). In the treatment with repeated additions of essential oil components, the extent of TCE degradation was increased considerably in all samples (Fig. 3d, e, and f). However, longer lag periods before TCE degradation were found with cumene- and limonene-induced cells (Fig. 3d and e). Both cumene and limonene were growth substrates for *Rhodococcus* sp. L4, and thus they were rapidly utilized prior to TCE degradation. Cumin aldehyde-induced cells degraded TCE almost completely within 2 h, while the amount of cumin aldehyde increased after the third addition (Fig. 3f). This was probably because a high concentration of cumin aldehyde is toxic to bacterial cells (24). In the control experiment with killed cells, the reduction of TCE was insignificant, while the amount of essential oil component increased over time (Fig. 3g, h, and i). Consequently, abiotic processes, such as TCE adsorption to the bacterial cells and the interaction between TCE and each essential oil, had no impact on TCE loss.

Immobilization of *Rhodococcus* sp. L4 on plant materials rich in essential oils. The immobilization of *Rhodococcus* sp. L4 on cumin seeds, orange peels, and lemongrass leaves increased the TCE removal efficiency, while the efficiency of cells immobilized on loofah sponge and activated carbon remained the same after immobilization (see Fig. S1 in the supplemental material). These results confirmed that plant essential oils were essential for the induction of TCE-degrading enzymes in *Rhodococcus* sp. L4. Since lemongrass leaves adsorbed large amounts of TCE and orange peels deteriorated rapidly, cumin seeds were selected as the immobilizing material for further TCE degradation tests. The inoculated cumin seeds contained large numbers of *Rhodococcus* sp. L4 cells on their porous surfaces (Fig. 4a). In addition, EPS fibers were present between the cells (Fig. 4a, panel 3). The largest amount of EPS was observed when the bacteria were immobilized on cumin seeds with a 500-μm to 1-mm particle size for 4 days (see Fig. S2 in the supplemental material). Thus, the subsequent TCE biodegradation tests were carried out with cumin seed-immobilized cells prepared under these conditions.

Efficiency of TCE degradation by *Rhodococcus* sp. L4 immobilized on cumin seeds. *Rhodococcus* sp. L4 immobilized on cumin seeds was able to degrade TCE at all initial concentrations tested (2 to 68 μM), and the largest amount of TCE degraded was 270 nmol, corresponding to about 2.5 times the amount degraded by the suspended cells (Fig. 1). The initial specific rate (k_c) of TCE degradation was calculated as described by Alvarez-Cohen and McCarty (1). The k_c of immobilized cells (R^2 = 0.82) was 0.0046 mg TCE mg $cells^{-1}$ day^{-1} (1.9 nmol mg of $protein^{-1}$ min^{-1}) in the first 5 days (Fig. 4b, inset), while the k_c of suspended cells induced with cumin essential oil was 0.094 mg TCE mg $cells^{-1}$ day^{-1} (24). When TCE was added only at the beginning, about 110 nmol TCE was removed by immobilized cells, while about 10 nmol TCE was absorbed by the immobilizing materials after 5 days of incubation (Fig. 4b, inset). To determine whether the immobilized cells could degrade TCE continuously, TCE was repeatedly added to the cultures, on days 4, 5, and 6. As a result of 4 additions, the total

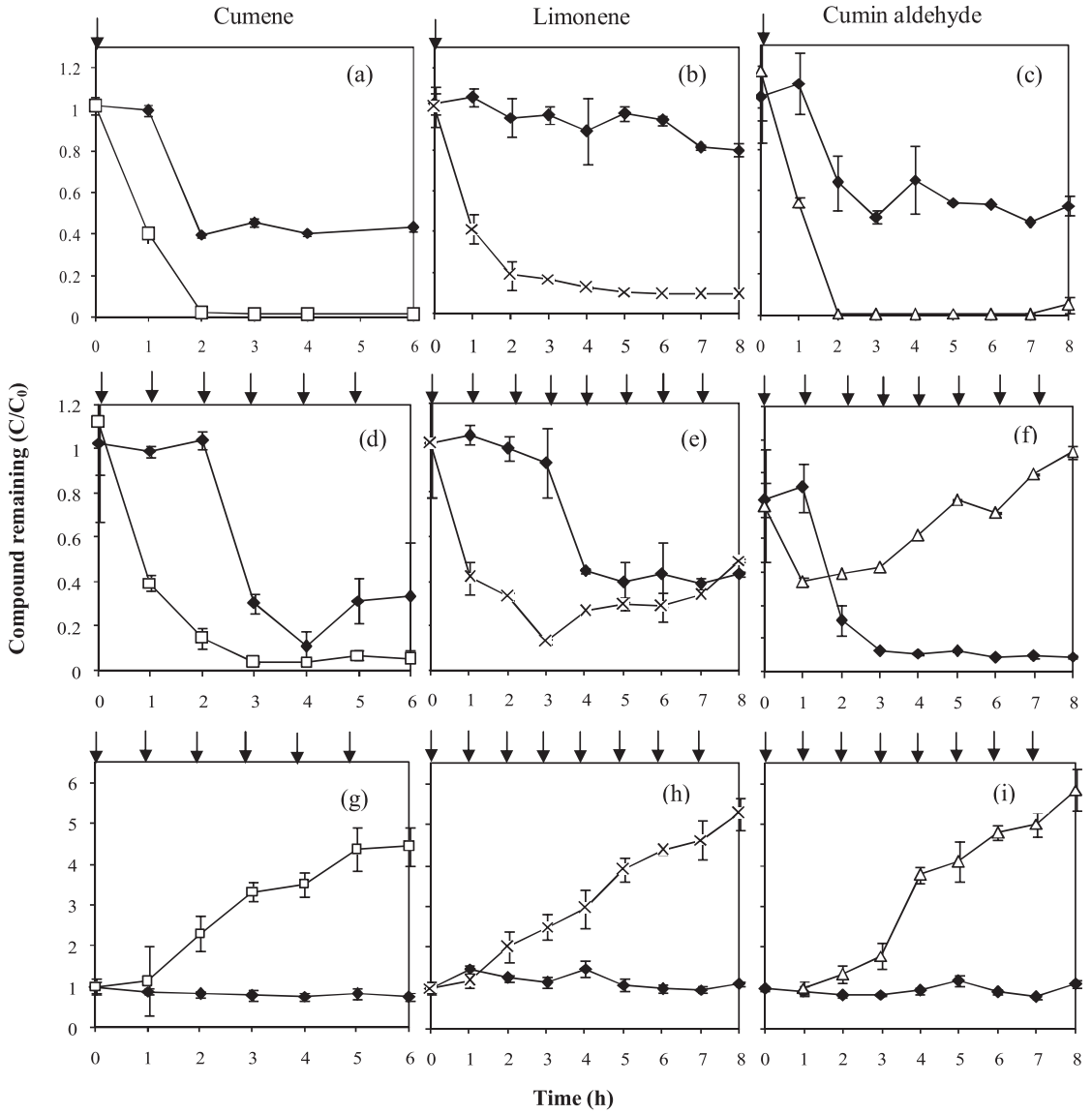

FIG. 3. Degradation of TCE (◆), cumene (□), limonene (×), and cumin aldehyde (△) by resting (a to f) and killed (g to i) cells of *Rhodococcus* sp. L4. The bacteria were induced with each oil component prior to TCE biodegradation assay. The arrows show the repeated addition of essential oil components to the samples. The amount of oil component at each time point was measured before the addition of new oil components. TCE was added only at the beginning of the study.

amount of TCE degraded was about 430 nmol, corresponding to about four times the amount degraded by suspended cells (Fig. 4). TCE removal by killed immobilized cells on cumin seeds, representing TCE loss by abiotic factors, was insignificant at the beginning, but the loss became larger at the end of study, when about 160 nmol had disappeared (Fig. 4).

Reuse of *Rhodococcus* sp. L4 immobilized on cumin seeds for TCE degradation. The results described above showed that *Rhodococcus* sp. L4 was inhibited and that its enzymes were inactivated during TCE degradation (Fig. 1 and 2). Therefore, it was necessary to reactivate the bacteria after TCE degradation. The used immobilized cells were reactivated by incubation in fresh MS medium for 12 to 24 h before they were reused in another TCE biodegradation experiment. During the first and second applications, immobilized cells degraded about 60 to 110 nmol TCE, while 10 to 20 nmol of TCE was adsorbed on control materials (Fig. 5). However, the samples without reactivation degraded smaller amounts of TCE than the reactivated samples in the third application (Fig. 5). During the first application, the number of toluene/TCE degraders on cumin seeds was 9.4×10^{10} CFU g seed^{-1} at the beginning and increased to 2.9×10^{11} CFU g seed^{-1} after 5 days of incubation. The numbers of bacteria after the second and third application cycles were about the same, at 0.9×10^{13} to 1.0×10^{13} CFU g seed^{-1}. In liquid medium, the number of toluene/TCE degraders after each application was 2.5×10^{6} to 920×10^{6} CFU of ml^{-1}.

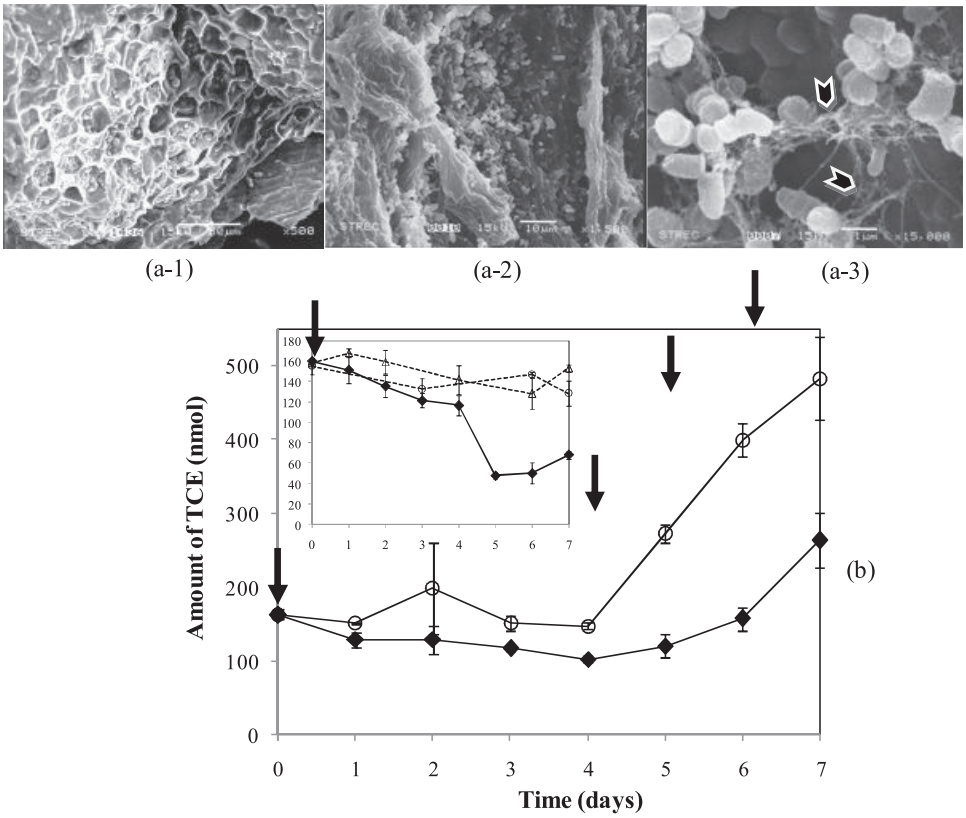

FIG. 4. *Rhodococcus* sp. L4 attached to the porous surfaces of cumin seeds after 4 days of incubation, as visualized by scanning electron microscopy (arrows indicate EPS fibers) (a, panels 1 to 3), and its TCE degradation efficiency (◆), determined with single (b, inset) and repeated (b) TCE additions. The control treatments were killed immobilized cells (○) and uninoculated materials (△). In the experiment with repeated TCE additions, 160 nmol TCE was injected into the vials after initial TCE degradation, and the same amount was injected every day, as indicated by the arrows. The aqueous concentration of TCE was kept in the range of about 50 μM to avoid dose-dependent substrate inhibition.

DISCUSSION

Several groups have reported that TCE or its oxidation products can inhibit or inactivate TCE-degrading bacteria and enzymes (11, 13, 18, 19, 27). Similarly, TCE transformation had a negative effect on the activities of *Rhodococcus* sp. L4 and its TDO. TDO activity of *Rhodococcus* sp. L4 was reversibly inhibited, since this activity was recovered after TCE was removed from the system. Nevertheless, the slightly lower activity of recovered TDO suggested that a small part of the enzyme was inactivated or damaged irreversibly during TCE degradation. We are aware that there may have been more than one oxygenase in the extracts. However, since activity was completely inhibited by higher concentrations of TCE and since most of the activity could be recovered, this must also include the TCE converting enzyme. To overcome this inactivation, energy-generating substrates such as limonene, cumene, and cumin aldehyde were repeatedly added to the cells of *Rhodococcus* sp. L4. The results showed that TCE degradation was enhanced and that about 96 to 144 nmol of 14 μM TCE was degraded. TCE probably acted more like an inhibitor than like a true substrate for *Rhodococcus* sp. L4 TDO. This phenomenon is similar to the case for the enzyme from *Pseudomonas putida* F1 (18). The role of the essential oil molecules in this study was probably to induce new TCE-degrading enzyme during the experiment and to displace TCE or its intermediates from the active site of TDO for restoring TCE-degrading activity.

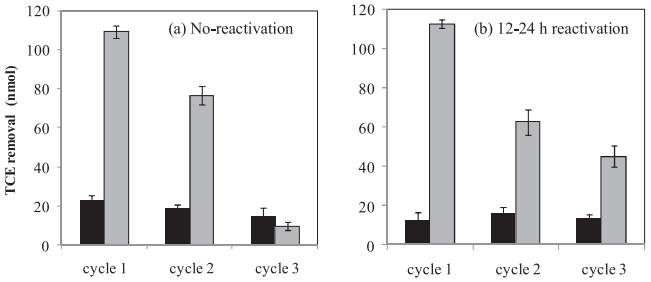

FIG. 5. TCE removal by cells immobilized on cumin seeds (gray bars) and by killed immobilized cells (black bars) in 3 application cycles. The samples consisted of nonreactivated and reactivated immobilized cells. Reactivation was carried out by submerging the used immobilized cells in new MS medium for 12 to 24 h. For each application cycle, the total mass of TCE was 160 nmol and the incubation period was 5 days.

Although the repeated addition of these essential oil components could enhance the TCE-degrading activity of *Rhodococcus* sp. L4, the use of this knowledge for TCE bioremediation may not be feasible because of their high volatility and low bioavailability. Therefore, the bacteria were immobilized on plant materials rich in essential oils, such as cumin seeds,

lemongrass leaves, and orange peels. These plants contain several essential oil components, such as cumene, cumin aldehyde, limonene, and citral, that have been found to stimulate TCE degradation in *Rhodococcus* sp. L4 (24). In addition, these selected plants are local agricultural products and are inexpensive. Of these materials, cumin seeds were the best for cell immobilization in terms of enhancing TCE-degrading efficiency and material stability. The strong attachment of cells to cumin seeds was assisted by EPS formation, which was probably stimulated by essential oils readily released from the ground seeds. Similarly, citronellol and cinnamaldehyde, the main essential oil components in *Cymbopogon nardus* and *Cinnamomum aromaticum* (cassia) extracts, respectively, stimulated EPS formation in *Escherichia coli* ATCC 33456 and *Pseudomonas aeruginosa* PAO1 (20). It was suggested that high concentrations of these compounds (>1.75 mM) activate a stress-induced response by increasing EPS production or by acting as a poly-L-lysine-like adhesive.

The cumin seed-immobilized cells removed much larger amounts of TCE than suspended cells did, especially at high TCE concentrations (>25 μM), which indicated that they were more resistant to TCE. The immobilized cells were able to degrade TCE after repeated additions. After four TCE addition cycles, the immobilized cells had removed 430 nmol of the total of 640 nmol of TCE. However, the activity of immobilized cells tended to decrease, as indicated by the TCE accumulation at the end of the experiment. These results suggested that the bacteria were partially protected from the toxicity of TCE and its intermediates. In addition, the initial TCE degradation rate of cumin seed-immobilized cells was relatively low compared to that of suspended cells (24). This was probably due to mass transfer limitations which led to a decrease in activity of immobilized cells (4, 25).

The TCE-degrading toluene dioxygenase was inactivated during TCE conversion, but the inactivation could be reversed by dialysis. Accordingly, the activity of immobilized cells, which had been inactivated partially during TCE degradation, could be reactivated by incubation in mineral salts medium without TCE for 12 to 24 h before further use. The essential oils, organic content, and other nutrients available in the seeds supported bacterial growth, as indicated by an increase in the number of toluene/TCE degraders during the test. Several groups showed the benefits of essential oil and its components on the growth of bacteria and on degradation of xenobiotic compounds such as TCE and polychlorinated biphenyls (PCBs) (12, 17, 24). Although the number of bacteria had increased after the second and third application cycles, the TCE degradation efficiency was decreased (Fig. 5). This may be because the enzymes involved in TCE degradation could not be recovered completely.

In summary, the degradation of TCE was found to have detrimental impacts on *Rhodococcus* sp. L4 and its enzyme, similar to the case in other TCE-degrading bacteria. However, *Rhodococcus* sp. L4 immobilized on cumin seeds maintained a higher TCE-degrading activity than that of suspended cells and thereby improved the overall TCE-degrading efficiency. Although the bacteria and their enzymes were still inactivated during TCE transformation, part of the activity could be recovered by simply submerging the used immobilized materials in MS medium to remove TCE and its degradation intermediates. Consequently, those immobilized cells could be used for continuous TCE degradation in bioreactors as well as in contaminated areas.

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